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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/85, 15/00		A1	(11) International Publication Number: WO 99/40213 (43) International Publication Date: 12 August 1999 (12.08.99)
(21) International Application Number: PCT/US99/02868 (22) International Filing Date: 9 February 1999 (09.02.99)		(81) Designated States: AU, CA, NZ, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(30) Priority Data: 60/073,386 9 February 1998 (09.02.98) US			
(71) Applicant: TRANXENOGEN, INCORPORATED [US/US]; Fuller Building, 222 Maple Avenue, Shrewsbury, MA 01545 (US).			
(72) Inventors: DITULLIO, Paul, A.; 42 Juniper Brook Road, Northboro, MA 01532 (US). EBERT, Karl, M.; 83 Elmwood Street, Millbury, MA 01527 (US).			
(74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).			
(54) Title: GENETIC MANIPULATION OF SPERMATOGONIA			
(57) Abstract The invention features a method of delivering DNA to a spermatogonium by infusing DNA <i>in situ</i> into a testicle of a non-human animal and administering a condition or substance to the testicle to increase uptake of DNA by the spermatogonium.			

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GENETIC MANIPULATION OF SPERMATOGONIA

Cross Reference To Related Applications

5 This application claims priority from provisional patent application serial number 60/073,386, filed on February 9, 1998, the contents of which are hereby incorporated by reference.

Background of the Invention

10 The invention relates to the production of transgenic animals.

The field of transgenics has grown rapidly since the initial experiments describing the introduction of foreign DNA into the developing zygote or embryo

15 (Brinster, R.L. et al., Proc. Natl. Acad. Sci. USA 82:4438-4442 (1985), Wagner et al., U.S. 4,873,191 (1989)). Transgenic technology has been applied to both laboratory and domestic species for the study of human diseases (see, e.g., Synder, B.W., et al., Mol. Reprod.

20 and Develop. 40:419-428 (1995)), production of pharmaceuticals in milk (see, for review article, Ebert, K.M. and J.P. Selgrath, "Changes in Domestic Livestock through Genetic Engineering" in *Applications in Mammalian Development*, Cold Spring Harbor Laboratory Press, 1991.),

25 develop improved agricultural stock (see, e.g., Ebert, K.M. et al., Animal Biotechnology 1:145-159 (1990)) and xenotransplantation (see, e.g., Osman, N., et al., Proc. natl. Acad. Sci USA 94:14677-14682 (1997)). However, the technique is limiting in that it only allows for the

30 addition of genetic material to the developing embryo and not the deletion or modification of the endogenous genes.

In addition, the microinjection of DNA into the nucleus is an inefficient process resulting in only 1-2% transgenic offspring from embryos injected and frequently 35 producing mosaic animals which do not have the transgene in all cells.

- 3 -

preparation is free of viral proteins or particles, DEAE-dextran, phospholipids, lipids, or calcium phosphate. The DNA contains a sequence encoding a selectable marker such as DNA encoding an antibiotic resistance gene, a 5 cell surface antigen, or thymidine kinase. Following administration of the DNA, transformed or transfected cells are selected by administering an antibiotic, antibody-toxin complex, or chemical agent either systemically or locally to the testes to kill cells which 10 do not express the DNA or to identify cells which express the DNA. The terms "transform" and "transfect" are used interchangeably throughout the specification; these terms refer to means of transferring or delivering DNA to a cell. A "transfected" or "transformed" cell is one that 15 contains the DNA sought to be delivered to it. The DNA may also contain a second promoter which directs expression of an apoptotic gene to selectively kill germ cells which have not undergone homologous recombination with the administered DNA. The DNA is administered in a 20 volume of solution sufficient to infuse the entire testicle, e.g., 0.1 ml per testicle/per treatment for a newborn animal and up to 5 ml per testicle/per treatment for an adult animal. Repeated treatments may be carried out if desired, e.g., to increase DNA uptake.

25 Preferably, the non-human animal is a sheep, goat, pig, cow, chicken, rabbit, rat, mouse, or guinea pig. More preferably, the animal is prepubetal, e.g., at an age at which the testicle has not yet begun to produce sperm. For example, the preferred age of a pig is at 30 least 30 days but not greater than 100 days. At this age, the number of target cells, i.e., spermatogonia, is relatively low. An advantage of this approach is that destruction of spermatogenic cells prior to administration of DNA is not required.

- 5 -

of porcine CD59 (a CD59 "knockout" pig) or the animal expresses human H-transferase and/or lacks porcine Gal(α1,3) galactosyl transferase.

The methods of the invention solve many ongoing problems in DNA delivery for the generation of transgenic animals. For example, an adenovirus genome can only carry a transgene of limited size. The use of sperm does not limit the transgene size. For example, DNA as small as a few base pairs (bp) and as large as 100-200 kilobases (kb) are delivered using the methods of the invention. Typically, approximately 5 kb, 10 kb, 20 kb or 25 kb are delivered to target cells. However, up to 400 kb may be transferred to cells. For example, more than one vector or DNA fragment is transfected simultaneously to allow for the production of multimeric proteins (i.e., immunoglobulins, FAb fragments, fibrinogen, and collagen) or expression of more than one protein coding sequence.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1A is a diagram of a cross-section of a seminiferous tubule in a mammalian testes.

Fig. 1B is a diagram showing the organization of different cell types within the seminiferous tubules and the respective cell types.

Fig. 2 is a diagram showing the stages of spermatogenesis.

30 Detailed Description

Transgenic animals made using the methods described herein are used for xenotransplantation, pharmaceutical production, protein production, and the study of human diseases.

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electroejaculator, heart defibrillator, or any suitable source of electrical current. After the animal has recovered, semen is collected and analyzed for the presence of the transgene. Once the transgene has been 5 detected, the male will be bred to females to produce transgenic offspring. The time between infusion of the DNA and breeding varies depending on such factors as the age of the animal at infusion, age of sexual maturity, and the time required for differentiation from 10 stem/progenitor cell to sperm cell.

Direct delivery of DNA to sperm cells/spermatogonia

The invention described herein is a method for producing a non-human transgenic animal by genetically manipulating the stem cells/spermatogonia of the male 15 testes *in vivo*. The testes are composed of a series of tightly coiled tubes, termed seminiferous tubules. Testes are derived from two cell types; cells of the mesonephros region form the structural cells and the primordial germ cells give rise to the spermatogonia 20 (Figs. 1A and B). The seminiferous tubules contain the stem cells (spermatogonia) which when properly stimulated will undergo mitosis and meiosis to form mature sperm allowing reproduction (Fig. 2). Spermatogenesis begins in puberty and continues throughout the adult life. 25 Therefore, the spermatogonia of the male represent the only truly regenerating stem cell population in mammals, and the introduction of DNA into a spermatogonia results in a continuous production of transgenic sperm. All transgenic animals produced by this technique carry the 30 transgene in their germline because the DNA is integrated in the sperm before fertilization. The DNA delivery method described herein is used to transfet the spermatogonia *in vivo*. The approach is also useful to transfet cells *in vitro*. For example, spermatagonia-

- 9 -

transgene, transgenic animals are produced by breeding the male to females in estrous and allowing the animals to deliver. The progeny are tested for the presence of the transgene by PCR or southern blot analysis.

5 The overall efficiency of the procedure is dependent on the method of gene delivery but can be increased in several ways such as 1) subjecting the testicle to multiple rounds of transfection with the DNA vector, or 2) adding a selectable marker, e.g., an
10 antibiotic resistance gene, to the DNA vector and infusing the testicle with the antibiotic or drug following transfection to select for the transgenic cells.

Example 1: Timing of Testes Transfection

15 Experiments were carried out to determine the optimal age of animal at which *in situ* DNA delivery to the testes is most efficient. As a male animal matures, the testes increase in size. The increase in size corresponds to an increase in the number of target cells
20 and primordial germ cells/spermatogonia. As a result, partial sterilization may be required prior to DNA delivery to mature animals.

According to the invention, the testes are transfected at an age when the seminiferous tubules are
25 open to allow the DNA access to the spermatogonia but before the testes begin to actively produce sperm by meiosis. Since different species mature at different rates, this age varies among species. The optimal age for any given species is determined using known methods,
30 e.g., monitoring a maturing animal for the onset of sperm production or histologically examining the testes from newborn to adult. Once the preferred age for DNA delivery is determined for a given species, this process need not be repeated for each individual to be treated.

- 11 -

successfully carried tracking dye. However, the larger size of the testicle at the adult stage may reduce efficiency of DNA delivery and transfection. These data indicate that the preferred age for DNA delivery and
5 transfection of the testicle of a Yorkshire boar is between 30 and 100 days of age.

Example 2: Transfection of Testes

The method described below is applicable to all species which reproduce through the production of sperm,
10 for example but not limited to mice, rats, rabbits, pigs, goats, sheep and cows. DNA is delivered to the area surrounding the spermatogonia by utilizing the seminiferous tubules as conduits to transport the genetic material throughout the testes.

15 To increase overall efficiency of DNA delivery, prepubetal animals are used (thereby reducing the number of target cells in the testes to be transfected). Use of non-viral DNA is preferable because this approach removes any size limitation on the vector. The protocol
20 described herein is useful for any DNA formulation (i.e., for either naked DNA or DNA in complex with compounds such as lipids or DEAE-dextran). Alternatively, a virus suitable to infect target cells of the testes (stem cells) is also a useful vehicle to deliver DNA to the
25 target cells.

The resulting phenotype of the animal produced by a transformed sperm is dependent on the type of the DNA introduced and is not dependent on the method being described. Introduction of a vector or transgene
30 containing mammary gland-specific regulatory elements directs expression of the desired protein in the animal's milk whereas a liver-specific promoter would target the blood. For example, the following tissue-specific promoters are used to direct preferential expression of
35 DNA in mammary gland tissue: goat beta casein promoter

- 13 -

the pig for analysis. The sperm are recovered from the semen by centrifugation, washed with phosphate buffered saline, and DNA isolated by digestion with protease followed by phenol extraction and ethanol precipitation.

5 The DNA isolated from the sperm is analyzed for the presence of the transgene by the PCR with primers specific for the transgene. Successful "knockout" of DNA sequences is also evaluated using PCR or other techniques known in the art.

10 If the sperm DNA tests positive for the gene of interest, transgenic animals are produced by breeding the pig to sows in heat, using the semen for artificial insemination, or doing in vitro fertilization with the sperm. The recipient females are allowed to farrow and
15 the progeny tested for the presence of the transgene (or knockout) with DNA isolated from ear tissue or blood by the polymerase chain reaction.

Other embodiments are within the following claims

- 15 -

9. The method of claim 1, wherein said non-human animal is selected from the group consisting of a sheep, goat, pig, cow, chicken, rabbit, rat, mouse, and guinea pig.

5 10. The method of claim 9, wherein said non-human animal is a pig.

11. The method of claim 1, wherein said animal is prepubetal.

12. The method of claim 1, wherein said DNA 10 comprises a sequence encoding a selectable marker.

13. The method of claim 12, wherein said selectable marker is selected from the group consisting of antibiotic resistance gene, a cell surface antigen, or thymidine kinase.

15 14. The method of claim 1, wherein DNA is administered to said testicle before the time at which sperm production is detected.

15. The method of claim 9, wherein the age of said pig is at least 30 days.

20 16. The method of claim 15, wherein the age of said pig is not greater than 100 days.

17. The method of claim 1, wherein said DNA is naked.

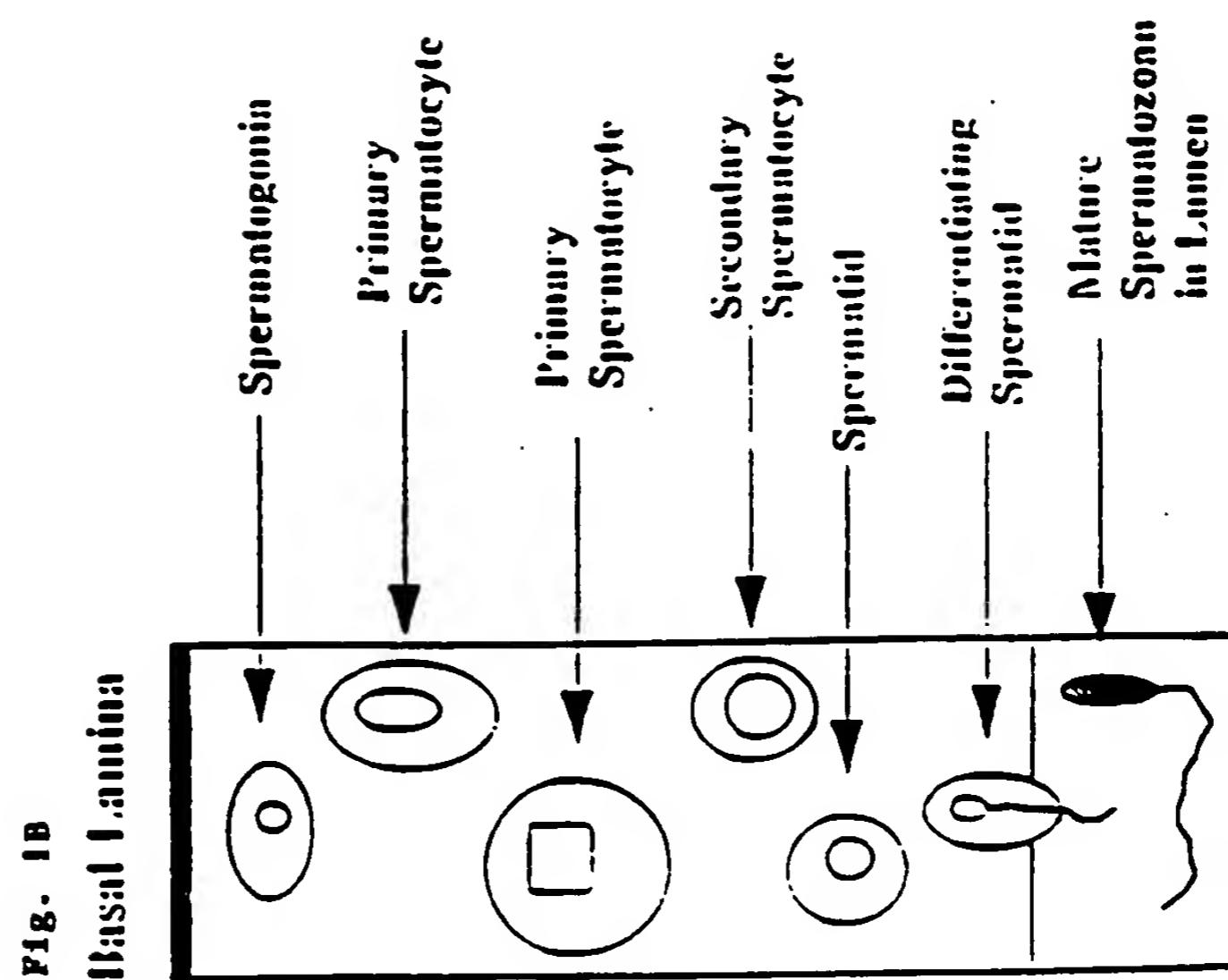
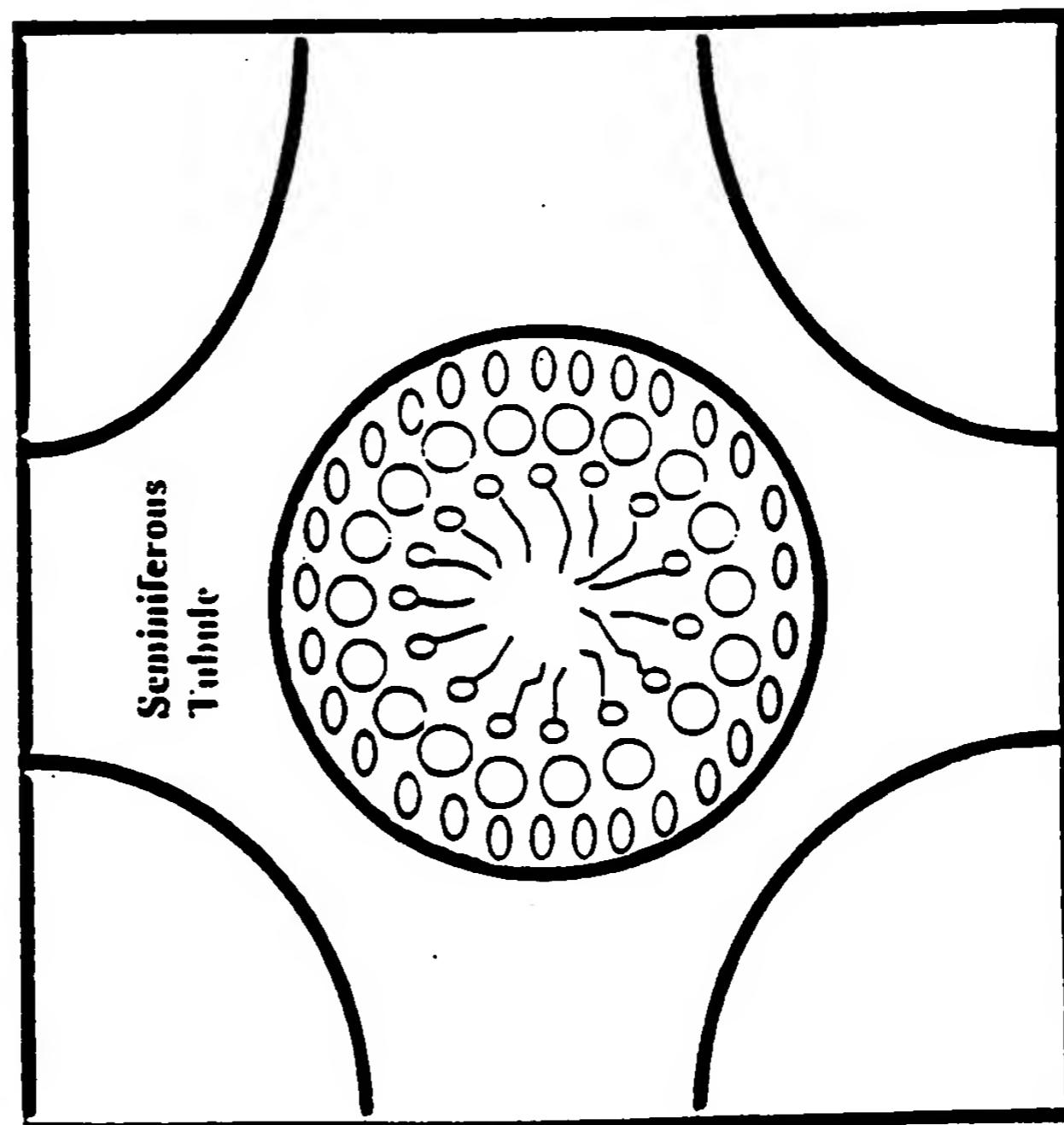


Fig. 1A



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02868

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/85, 15/00

US CL : 435/455; 800/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455; 800/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database BIOSIS on STN, Accession Number 1996:163441, OGAWA et al. Gene expression in blastocysts following direct injection of DNA into testis. Journal of Reproduction and Development. Abstract only, 1995, Vol. 41, No. 4, pages 379-382.	1,5,8,12,13 -----
Y	KIM et al. The development of the method for sperm-mediated gene transfer in mouse and pig. Theriogenology. 1996, Vol. 45, page 337, entire document.	1-20
X		1,5,9,10 -----
Y		1-20

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
O document referring to an oral disclosure, use, exhibition or other means	*E*	document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

09 APRIL 1999

Date of mailing of the international search report

06 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02868

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/85, 15/00		A1	(11) International Publication Number: WO 99/40213 (43) International Publication Date: 12 August 1999 (12.08.99)
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(30) Priority Data: 60/073,386 9 February 1998 (09.02.98) US		Published <i>With international search report.</i>	
<p>(71) Applicant: TRANXENOGEN, INCORPORATED [US/US]; Fuller Building, 222 Maple Avenue, Shrewsbury, MA 01545 (US).</p> <p>(72) Inventors: DITULLIO, Paul, A.; 42 Juniper Brook Road, Northboro, MA 01532 (US). EBERT, Karl, M.; 83 Elmwood Street, Millbury, MA 01527 (US).</p> <p>(74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).</p>			
<p>(54) Title: GENETIC MANIPULATION OF SPERMATOGONIA</p> <p>(57) Abstract</p> <p>The invention features a method of delivering DNA to a spermatogonium by infusing DNA <i>in situ</i> into a testicle of a non-human animal and administering a condition or substance to the testicle to increase uptake of DNA by the spermatogonium.</p>			

*(Referred to in PCT Gazette No. 42/1999, Section II)

BNSDOCID: <WO_9940213A1_IA>

- 1 -

GENETIC MANIPULATION OF SPERMATOGONIA

Cross Reference To Related Applications

5 This application claims priority from provisional patent application serial number 60/073,386, filed on February 9, 1998, the contents of which are hereby incorporated by reference.

Background of the Invention

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25 develop improved agricultural stock (see, e.g., Ebert, K.M. et al., Animal Biotechnology 1:145-159 (1990)) and xenotransplantation (see, e.g., Osman, N., et al., Proc. natl. Acad. Sci USA 94:14677-14682 (1997)). However, the technique is limiting in that it only allows for the

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preparation is free of viral proteins or particles, DEAE-dextran, phospholipids, lipids, or calcium phosphate. The DNA contains a sequence encoding a selectable marker such as DNA encoding an antibiotic resistance gene, a 5 cell surface antigen, or thymidine kinase. Following administration of the DNA, transformed or transfected cells are selected by administering an antibiotic, antibody-toxin complex, or chemical agent either systemically or locally to the testes to kill cells which 10 do not express the DNA or to identify cells which express the DNA. The terms "transform" and "transfect" are used interchangeably throughout the specification; these terms refer to means of transferring or delivering DNA to a cell. A "transfected" or "transformed" cell is one that 15 contains the DNA sought to be delivered to it. The DNA may also contain a second promoter which directs expression of an apoptotic gene to selectively kill germ cells which have not undergone homologous recombination with the administered DNA. The DNA is administered in a 20 volume of solution sufficient to infuse the entire testicle, e.g., 0.1 ml per testicle/per treatment for a newborn animal and up to 5 ml per testicle/per treatment for an adult animal. Repeated treatments may be carried out if desired, e.g., to increase DNA uptake.

25 Preferably, the non-human animal is a sheep, goat, pig, cow, chicken, rabbit, rat, mouse, or guinea pig. More preferably, the animal is prepubetal, e.g., at an age at which the testicle has not yet begun to produce sperm. For example, the preferred age of a pig is at 30 least 30 days but not greater than 100 days. At this age, the number of target cells, i.e., spermatogonia, is relatively low. An advantage of this approach is that destruction of spermatogenic cells prior to administration of DNA is not required.

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of porcine CD59 (a CD59 "knockout" pig) or the animal expresses human H-transferase and/or lacks porcine Gal(α1,3) galactosyl transferase.

The methods of the invention solve many ongoing problems in DNA delivery for the generation of transgenic animals. For example, an adenovirus genome can only carry a transgene of limited size. The use of sperm does not limit the transgene size. For example, DNA as small as a few base pairs (bp) and as large as 100-200 kilobases (kb) are delivered using the methods of the invention. Typically, approximately 5 kb, 10 kb, 20 kb or 25 kb are delivered to target cells. However, up to 400 kb may be transferred to cells. For example, more than one vector or DNA fragment is transfected simultaneously to allow for the production of multimeric proteins (i.e., immunoglobulins, FAb fragments, fibrinogen, and collagen) or expression of more than one protein coding sequence.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1A is a diagram of a cross-section of a seminiferous tubule in a mammalian testes.

Fig. 1B is a diagram showing the organization of different cell types within the seminiferous tubules and the respective cell types.

Fig. 2 is a diagram showing the stages of spermatogenesis.

30 Detailed Description

Transgenic animals made using the methods described herein are used for xenotransplantation, pharmaceutical production, protein production, and the study of human diseases.

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electroejaculator, heart defibrillator, or any suitable source of electrical current. After the animal has recovered, semen is collected and analyzed for the presence of the transgene. Once the transgene has been 5 detected, the male will be bred to females to produce transgenic offspring. The time between infusion of the DNA and breeding varies depending on such factors as the age of the animal at infusion, age of sexual maturity, and the time required for differentiation from 10 stem/progenitor cell to sperm cell.

Direct delivery of DNA to sperm cells/spermatagonia

The invention described herein is a method for producing a non-human transgenic animal by genetically manipulating the stem cells/spermatogonia of the male 15 testes *in vivo*. The testes are composed of a series of tightly coiled tubes, termed seminiferous tubules. Testes are derived from two cell types; cells of the mesonephros region form the structural cells and the primordial germ cells give rise to the spermatogonia 20 (Figs. 1A and B). The seminiferous tubules contain the stem cells (spermatogonia) which when properly stimulated will undergo mitosis and meiosis to form mature sperm allowing reproduction (Fig. 2). Spermatogenesis begins in puberty and continues throughout the adult life. 25 Therefore, the spermatogonia of the male represent the only truly regenerating stem cell population in mammals, and the introduction of DNA into a spermatogonia results in a continuous production of transgenic sperm. All 30 transgenic animals produced by this technique carry the transgene in their germline because the DNA is integrated in the sperm before fertilization. The DNA delivery method described herein is used to transfet the spermatogonia *in vivo*. The approach is also useful to transfet cells *in vitro*. For example, spermatagonia-

- 9 -

transgene, transgenic animals are produced by breeding the male to females in estrous and allowing the animals to deliver. The progeny are tested for the presence of the transgene by PCR or southern blot analysis.

5 The overall efficiency of the procedure is dependent on the method of gene delivery but can be increased in several ways such as 1) subjecting the testicle to multiple rounds of transfection with the DNA vector, or 2) adding a selectable marker, e.g., an
10 antibiotic resistance gene, to the DNA vector and infusing the testicle with the antibiotic or drug following transfection to select for the transgenic cells.

Example 1: Timing of Testes Transfection

15 Experiments were carried out to determine the optimal age of animal at which *in situ* DNA delivery to the testes is most efficient. As a male animal matures, the testes increase in size. The increase in size corresponds to an increase in the number of target cells
20 and primordial germ cells/spermatogonia. As a result, partial sterilization may be required prior to DNA delivery to mature animals.

According to the invention, the testes are transfected at an age when the seminiferous tubules are
25 open to allow the DNA access to the spermatogonia but before the testes begin to actively produce sperm by meiosis. Since different species mature at different rates, this age varies among species. The optimal age for any given species is determined using known methods,
30 e.g., monitoring a maturing animal for the onset of sperm production or histologically examining the testes from newborn to adult. Once the preferred age for DNA delivery is determined for a given species, this process need not be repeated for each individual to be treated.

- 11 -

successfully carried tracking dye. However, the larger size of the testicle at the adult stage may reduce efficiency of DNA delivery and transfection. These data indicate that the preferred age for DNA delivery and
5 transfection of the testicle of a Yorkshire boar is between 30 and 100 days of age.

Example 2: Transfection of Testes

The method described below is applicable to all species which reproduce through the production of sperm,
10 for example but not limited to mice, rats, rabbits, pigs, goats, sheep and cows. DNA is delivered to the area surrounding the spermatogonia by utilizing the seminiferous tubules as conduits to transport the genetic material throughout the testes.

15 To increase overall efficiency of DNA delivery, prepubetal animals are used (thereby reducing the number of target cells in the testes to be transfected). Use of non-viral DNA is preferable because this approach removes any size limitation on the vector. The protocol
20 described herein is useful for any DNA formulation (i.e., for either naked DNA or DNA in complex with compounds such as lipids or DEAE-dextran). Alternatively, a virus suitable to infect target cells of the testes (stem cells) is also a useful vehicle to deliver DNA to the
25 target cells.

The resulting phenotype of the animal produced by a transformed sperm is dependent on the type of the DNA introduced and is not dependent on the method being described. Introduction of a vector or transgene
30 containing mammary gland-specific regulatory elements directs expression of the desired protein in the animal's milk whereas a liver-specific promoter would target the blood. For example, the following tissue-specific promoters are used to direct preferential expression of
35 DNA in mammary gland tissue: goat beta casein promoter

- 13 -

the pig for analysis. The sperm are recovered from the semen by centrifugation, washed with phosphate buffered saline, and DNA isolated by digestion with protease followed by phenol extraction and ethanol precipitation.

- 5 The DNA isolated from the sperm is analyzed for the presence of the transgene by the PCR with primers specific for the transgene. Successful "knockout" of DNA sequences is also evaluated using PCR or other techniques known in the art.
- 10 If the sperm DNA tests positive for the gene of interest, transgenic animals are produced by breeding the pig to sows in heat, using the semen for artificial insemination, or doing in vitro fertilization with the sperm. The recipient females are allowed to farrow and
15 the progeny tested for the presence of the transgene (or knockout) with DNA isolated from ear tissue or blood by the polymerase chain reaction.

Other embodiments are within the following claims

- 15 -

9. The method of claim 1, wherein said non-human animal is selected from the group consisting of a sheep, goat, pig, cow, chicken, rabbit, rat, mouse, and guinea pig.

5 10. The method of claim 9, wherein said non-human animal is a pig.

11. The method of claim 1, wherein said animal is prepubetal.

12. The method of claim 1, wherein said DNA
10 comprises a sequence encoding a selectable marker.

13. The method of claim 12, wherein said selectable marker is selected from the group consisting of antibiotic resistance gene, a cell surface antigen, or thymidine kinase.

15 14. The method of claim 1, wherein DNA is administered to said testicle before the time at which sperm production is detected.

15. The method of claim 9, wherein the age of said pig is at least 30 days.

20 16. The method of claim 15, wherein the age of said pig is not greater than 100 days.

17. The method of claim 1, wherein said DNA is naked.

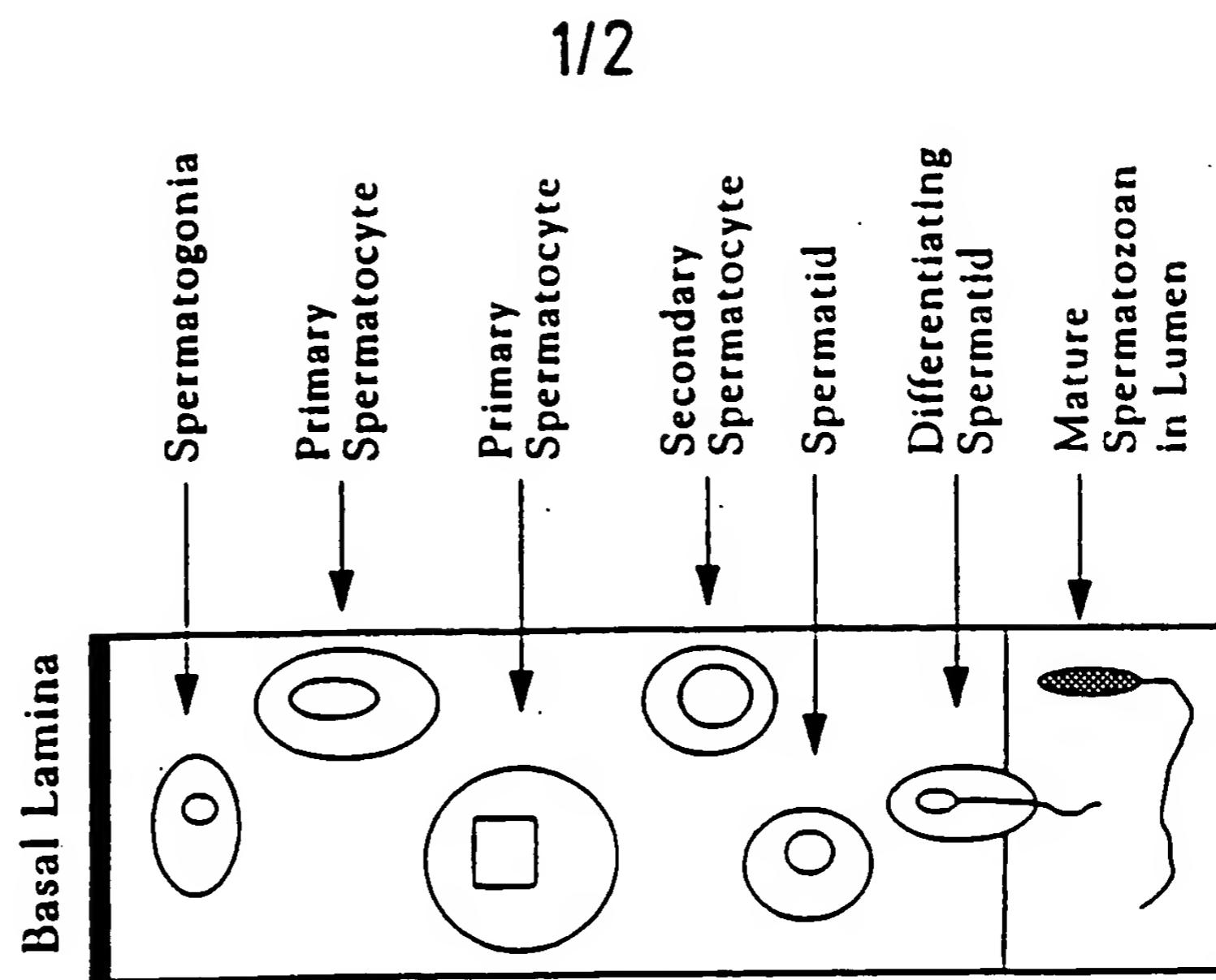


FIG. 1B

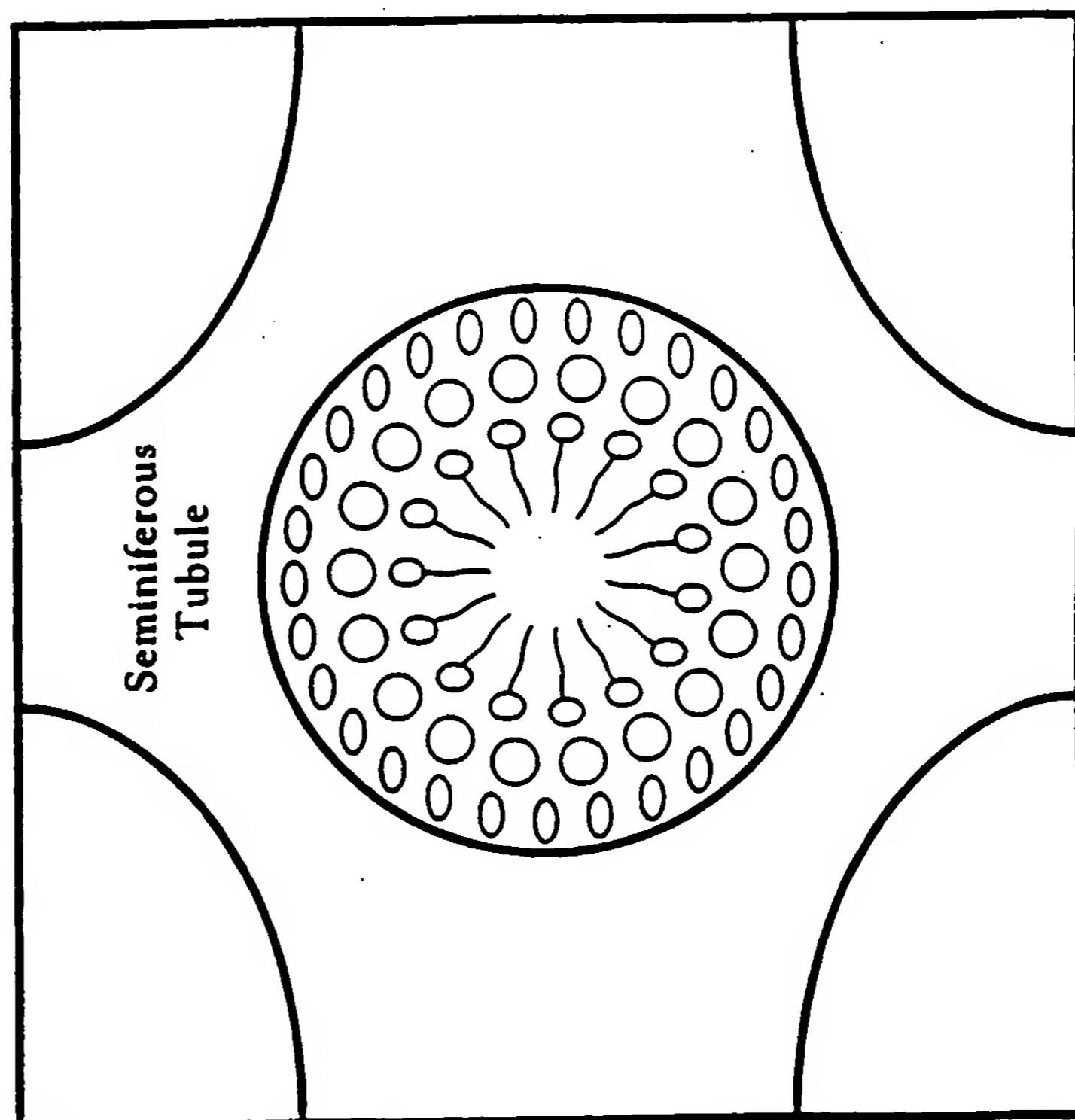


FIG. 1A

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02868

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/85, 15/00

US CL : 435/455; 800/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455; 800/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database BIOSIS on STN, Accession Number 1996:163441, OGAWA et al. Gene expression in blastocysts following direct injection of DNA into testis. Journal of Reproduction and Development. Abstract only, 1995, Vol. 41, No. 4, pages 379-382.	1,5,8,12,13 -----
-		1-20
Y		
X	KIM et al. The development of the method for sperm-mediated gene transfer in mouse and pig. Theriogenology. 1996, Vol. 45, page 337, entire document.	1,5,9,10 -----
-		
Y		1-20

Further documents are listed in the continuation of Box C.

See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

09 APRIL 1999

Date of mailing of the international search report

06 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02868

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.